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BRIEF REPORT

Cardiac Specific Gene Expression Changes in Long Term Culture of Murine Mesenchymal Stem Cells

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Murine MSCs are a readily available source of adult stem cells enabling extensive *in vitro* study of this cell population. MSCs have been described as multipotent, and have been proven capable of differentiation into several connective tissue types. Furthermore some studies have suggested an ability to differentiate into non-connective tissue cell types such as the cardiomyocyte. The aim of this study was to differentiate murine MSCs toward cardiac lineage with the commonly used method of culture with 5' Azacytidine. Critically, baseline analysis of gene expression of passage four MSCs demonstrated expression of key cardiac markers including cardiac troponin T and I, and the ryanodine receptor. Furthermore, expression analysis of these genes changed with time in culture and passage number. However, there was no significant alteration when cells were subjected to a differentiation protocol. This study therefore highlights the importance of analyzing baseline cells extensively, and indicates the limitations in extrapolating data for comparison between species. Furthermore this data brings into question the efficacy of cardiac differentiation using MSCs.

Keywords: Mesenchymal stem cell, Murine, Marker expression, Differentiation, Cardiac

Mesenchymal stem cells (MSCs) are defined using three key parameters; firstly adherence to tissue culture plastic, a simple and effective method of cell isolation; secondly conforming to specific surface antigen expression and finally, ability for multipotent differentiation *in vitro* (1). In recent years, aided by ease of isolation and culture, this cell population has been extensively studied in relation to use of these adult stem cells as a therapeutic agent and source of cellular precursors for tissue regeneration.

Rodent modeling has created the foundation for research in this field, however characterization of MSCs is challenging given the wealth of markers available (2). A number of studies across several species have shown that MSCs are capable of differentiating along three standard routes namely osteoblast, adipocyte, and chondrocyte cell types (2-7). Furthermore there is a suggestion that these mesodermal lineage cells have the ability to differentiate into non-connective tissue cell types such as neuronal, hepatocyte and cardiomyocyte (8-13).

In this study we obtained commercially available murine MSCs (Invitrogen) and characterized their marker expression following cardiac differentiation, and subsequently at different passage number. Murine MSCs were purchased from Invitrogen (Cat. No. 510502-01) and cultured in murine MSC media; DMEM low glucose containing Glutamax-I (Invitrogen), with 10% MSC qualified foetal bovine serum (FBS) (Invitrogen Cat. No. 12662-011) and 100 U/ml penicillin G and 100 μg/ml streptomycin (both Invitrogen). Cells were plated at a cell density of 5,000 cells per cm^2 at 37°C, 5% CO_2 and exhibited a typical MSC morphology with flattened adherent cells with multi-

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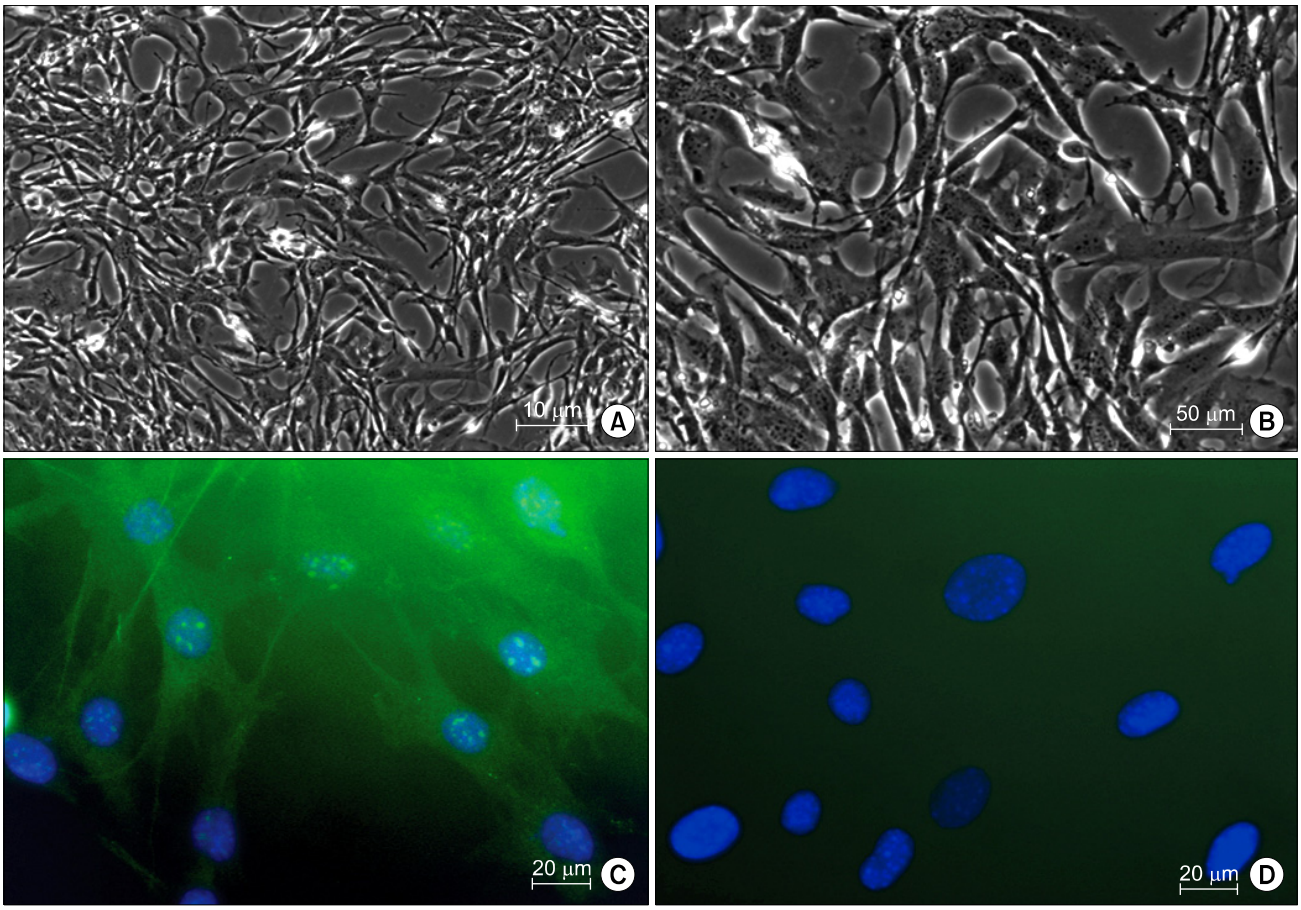

Fig. 1. Morphology and CD44 staining of Murine MSCs. Murine MSCs resembled other described MSCs in culture, with flattened morphology, and multiple jagged projections (A, magnification ×200 and B, magnification ×400). Murine MSCs were positive for *CD44* (C, magnification ×400). Negative controls were run alongside using secondary antibody alone (D, magnification ×400).

ple jagged projections (Fig. 1A, B). Cells were passaged every 3∼4 days, and no evidence of senescence was observed until passage 18 when experiments were stopped; and no difference in cell morphology or passage behaviour was noted. Cells were characterized and found to be *CD44* positive by both immunofluorescence (Fig. 1C, D) and RT-PCR (Fig. 2) and *CD45* negative by RT-PCR (data not shown). Total RNA was extracted from cell pellets using the RNeasy® mini-kit (Qiagen), and 500 ng reverse transcribed using Omniscript® RT (Qiagen). Primer pairs for murine markers were designed based on published sequences (NCBI) using Primer 3 software (http://primer3.sourceforge.net/) (Table 1). Bands of corresponding size were sequenced and compared to the genome. Immunofluorescence was performed on early passage monolayer MSCs grown on 4-well chambered slides (Corning) using rabbit polyclonal to *CD44* (Abcam, Ab65829) at 1 : 200, and secondary antibody Alexaflour 488 goat anti-rabbit IgG (H+L) (Invitrogen, A-11034) at 1 : 500.

Murine MSCs were exposed to a cardiac differentiation protocol using the demethylating agent 5' Azacytidine (5' AZA). 5×10^4 cells in 0.5 ml media was added to each well of a 24-well plate with standard MSC media, and 3 μM 5'AZA (Sigma) was added to the differentiated wells every 3 days for 3 weeks. Harvests of triplicate wells were taken at days 0, 7, 14 and 21 and RNA was subsequently extracted, and used for semi-quantitative RT-PCR using 100 ng RNA (Fig. 2). Vascular endothelial growth factor expression appeared to remain high and unchanged during the differentiation protocol in both test and control MSCs, compared to day 0. *NKx2.5* remained at a similar expression level to day 0 across all the samples except for day 21 undifferentiated, where expression levels appeared lower. *GATA 4* expression increased in differentiated samples over time. *Flk-1* appeared to have higher expression levels in differentiated samples at all time points compared to day 0 and control cells. Smooth muscle actin (*SMA*) appeared to decrease in all samples when com-

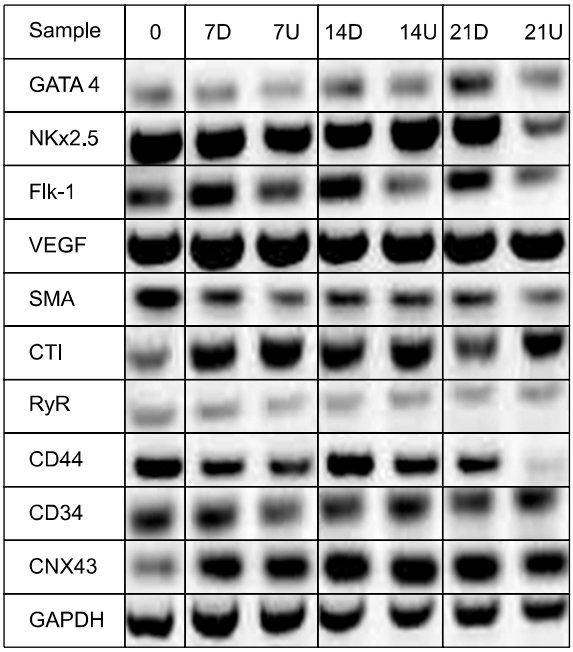

Fig. 2. Marker expression analysis of Murine MSCs during cardiac directed differentiation. Semi-quantitative RT-PCR analysis of markers expressed at time points of days 0, 7, 14 and 21 were compared against differentiated (D) and undifferentiated (U) cells. *VEGF*: vascular endothelial growth factor; *SMA*: smooth muscle actin; *CTI*: cardiac troponin I; *RyR*: cardiac ryanodine receptor; *CNX43*: Conexin 43.

pared to day 0, with a more pronounced drop in expression seen in the undifferentiated samples at all time-points. Cardiac troponin I (*CTI*) appeared to increase in all samples when compared to day 0. The ryanodine receptor was expressed at very low levels in all samples. *CD44* was expressed in all samples, with a slightly lower expression in undifferentiated samples compared to differentiated, and almost no expression seen in day 21 undifferentiated samples. Connexin 43 (*CNX 43*) expression appeared to be higher in all differentiated and control cells when compared to day 0. Murine MSCs did not express *c-kit*, a commonly used cardiac stem cell marker, or the β_1-adrenergic receptor (*Beta-1 Ad*; data not shown).

Our results suggested that murine MSCs may be altering expression pattern over time, we therefore investigated the effects of passage number on the murine MSCs by comparing early (passage 4) and late passage (passage 18) cells. No alteration in morphology was seen over the time period and there was no suggestion of senescence, with cell numbers continuing to increase by a magnitude of 2～3, even by the later passages. Using 500 ng total RNA and semi-quantitative RT-PCR it was seen that several markers demonstrated alterations in expression level (Fig. 3). Firstly it was noted that in this panel of markers we now saw both *islet 1* and cardiac troponin T (*CTT*) expression, which had not been seen in the day 0 MSCs in the differentiation experiment. This may be explained by the five fold increase in total RNA used in the passage difference

Table 1. Murine oligonucleotides

Mouse marker	Sequence 5′-3′	Predicted product size
C-Kit	F[a]: AGG GAT TCC CGG AGC CCA CA R[b]: GGG CCT GGA TTT GCT CTT TAA ATG C	252
Islet 1	F: GGT TTC TCC GGA TTT GGA AT R: CAC GAA GTC GTT CTT GCT GA	183
NKx2.5	F: GTG AAA CCT GCG TCG CCA CCA T R: TAG ACC TGC GCC TGC GAG AAG A	469
GATA 4	F: CAA GAT GAA TGG CAT CAA CC R: GGT TTG AAT CCC CTC TTT CC	216
Flk 1	F: AAG GCG CTG CTA GCT GTC GC R: TCC CGC TGT CCC CTG CAA GT	164
Cardiac troponin T	F: TGT CCA ACA TGA TGC ACT TTG GAG G R: GCT CCT TGG CCT TCT CTC TCA GT	165
B-adrenergic receptor	F: TCC TTC TAC GTG CCC CTG TGC A R: CGC TGG AAA GCC TTG CGA AGT	440
Cardiac ryanodine receptor	F: GTT CTG CAG TGC ACG GCG ACC R: GGC CTC CAC CTT GAG CAG TCT TCA T	268
Cardiac troponin I	F: CAG CGA TGC GGC TGG GGA AC R: CGA GCG TGA AGC TGT CGG CA	297
Vascular endothelial growth factor	F: GTG CAC TGG ACC CTG GCT TT R: CCG CAT GAT CTG CAT GGT GAT GT	300

Table 1. Continued

Mouse marker	Sequence 5′-3′	Predicted product size
Von willibrands factor	F: GGG TCT GCA ACT GCC CAC CC R: GTG GGG CCC AAT GTT GGC CT	160
Smooth muscle actin	F: GCC CAG CCA GTC GCT GTC AG R: TTA CTC CCT GAT GTC TGG GAC GTC C	180
CD44	F: AGC ACC TTG GCC ACC ACT CCT A R: AGC TGC AGT AGG CTG AAG GGT T	306
CD34	F: TGG CCC AGG GTA TCT GCC TGG R: GCT GGG AAG TTC TGT GCT ATT GGC C	212
CD45	F: CCT TAC CTG CTC GCA CCA CTG AA R: GCT TGC AAG GCC CAG AGT GGA T	419
Connexin 43	F: ATG AGC AGT CTG CCT TTC GT R: TCT GCT TCA AGT GCA TGT CC	249
GAPDH	F: CAT CAA CGG GAA GTC CAT CT R: GTG GAA GCA GGG ATG ATG TT	428
C-Kit	F[a]: AGG GAT TCC CGG AGC CCA CA R[b]: GGG CCT GGA TTT GCT CTT TAA ATG C	252
Islet 1	F: GGT TTC TCC GGA TTT GGA AT R: CAC GAA GTC GTT CTT GCT GA	183
NKx2.5	F: GTG AAA CCT GCG TCG CCA CCA T R: TAG ACC TGC GCC TGC GAG AAG A	469
GATA 4	F: CAA GAT GAA TGG CAT CAA CC R: GGT TTG AAT CCC CTC TTT CC	216
Flk 1	F: AAG GCG CTG CTA GCT GTC GC R: TCC CGC TGT CCC CTG CAA GT	164
Cardiac troponin T	F: TGT CCA ACA TGA TGC ACT TTG GAG G R: GCT CCT TGG CCT TCT CTC TCA GT	165
B-adrenergic receptor	F: TCC TTC TAC GTG CCC CTG TGC A R: CGC TGG AAA GCC TTG CGA AGT	440
Cardiac ryanodine receptor	F: GTT CTG CAG TGC ACG GCG ACC R: GGC CTC CAC CTT GAG CAG TCT TCA T	268
Cardiac troponin I	F: CAG CGA TGC GGC TGG GGA AC R: CGA GCG TGA AGC TGT CGG CA	297
Vascular endothelial growth factor	F: GTG CAC TGG ACC CTG GCT TT R: CCG CAT GAT CTG CAT GGT GAT GT	300
Von willibrands factor	F: GGG TCT GCA ACT GCC CAC CC R: GTG GGG CCC AAT GTT GGC CT	160
Smooth muscle actin	F: GCC CAG CCA GTC GCT GTC AG R: TTA CTC CCT GAT GTC TGG GAC GTC C	180
CD44	F: AGC ACC TTG GCC ACC ACT CCT A R: AGC TGC AGT AGG CTG AAG GGT T	306
CD34	F: TGG CCC AGG GTA TCT GCC TGG R: GCT GGG AAG TTC TGT GCT ATT GGC C	212
CD45	F: CCT TAC CTG CTC GCA CCA CTG AA R: GCT TGC AAG GCC CAG AGT GGA T	419
Connexin 43	F: ATG AGC AGT CTG CCT TTC GT R: TCT GCT TCA AGT GCA TGT CC	249
GAPDH	F: CAT CAA CGG GAA GTC CAT CT R: GTG GAA GCA GGG ATG ATG TT	428

[a]Forward primer, [b]reverse primer.

experiment, and therefore may indicate that RNA input is important when examining genes. Interestingly murine MSCs expressed *CD34*. It is generally accepted that MSCs should not express *CD34* (1), however differences in *CD34* expression has been observed in murine MSC populations, with interesting variation in the angiogenic potential be-

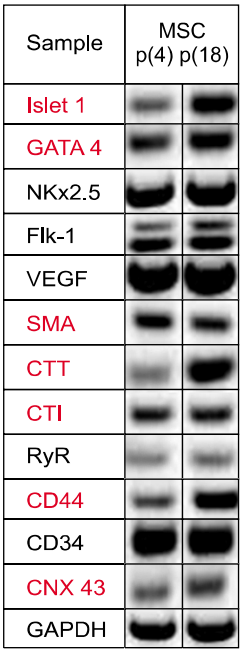

Fig. 3. Murine MSC marker expression changes with passage number. Murine MSCs were analysed using semi-quantitative RT-PCR for marker expression at early passage (passage 4; p(4)) and late passage (passage 18; p(18)). Markers with expression level variation noted with names in red. *VEGF*: vascular endothelial growth factor; *SMA*: smooth muscle actin; *CTT*: cardiac troponin T; *CTI*: cardiac troponin I; *RyR*: cardiac ryanodine receptor; *CNX43*: Conexin 43.

tween positive and negative populations (14). Furthermore we noted an increase in expression of *islet 1*, *GATA 4*, *CTI*, *CTT*, *CD44* and *CXN43*, and a decrease in the expression of *SMA* from early to late passage respectively. These results taken in isolation could suggest movement toward a cardiac lineage expression profile simply as a function of culture duration. Key differences in MSC expression of cardiac lineage genes may be inadvertently used as proof of differentiation when we take into consideration that murine MSCs used in this study expressed genes deemed to be indicative of a cardiac phenotype at baseline and levels of expression altered by duration of culture alone. Alteration in gene expression by passage number has been reported previously in murine MSCs (15) and is therefore an important finding when analyzing results.

These results highlight the importance of interpretation of differentiation data, and in defining differentiation criteria prior to the experiment particularly in the context of species type and passage number and illustrate the importance of appropriate controls. Rodent MSCs have been described capable of cardiac differentiation using 5' AZA based upon cardiac marker expression (16-18). This study aims to highlight the importance of fully analyzing baseline cells prior to experimentation and may warrant the need for global marker expression profiling studies. When making comparisons between MSC populations it is imperative to identify all confounding factors; species differences, site of MSC isolation, technique for isolation and passage number can all have an influence upon variation within MSC populations.

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Potential conflict of interest

The authors have no conflicting financial interest.

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